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Stability of Cryopreserved Samples of Mutant Mice

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Genetically modified animals are unique models with enormous scientific potential. Cryopreservation of pre-implantation embryos or of spermatozoa is a common approach to save those lines. The breeding of a line can be discontinued if a sufficient number of samples have been cryopreserved. To maintain the opportunity to recover a line, it is mandatory to assess the quality of the cryopreserved samples and to assure safe long-term storage conditions. Here, we investigated the revitalization rate of cryopreserved pre-implantation embryos stored *in-house* up to 158 months, of imported (and shipped) embryos, and of embryos received after *in vitro* fertilization. The storage period did not affect the revitalization rate, whereas the recovery of imported embryos was significantly reduced, possibly due to shipment conditions. The genotypes of genetically modified pups received following embryo-transfer were slightly smaller than expected by Mendelian laws. Intensive investigations of the hygienic state of the cryopreserved samples and the equipment used never showed microbiological contamination of a sample within a cryo-tube. However, environmental organisms were found frequently in the permanent freezers and dry shippers used. Since such contamination cannot be completely excluded and an embryo-transfer might not lead in all cases to a secure rederivation, foster mothers and revitalized pups should be housed in an intermediate facility and their health assessed before introducing them into the target facility.

Introduction

To obtain meaningful data in biomedical research, experiments using model organisms are indispensable. Mice are the most important laboratory animals used in biomedical research. Increasingly more important are genetically modified (GM) mouse lines—animals carrying one or more defined mutations in their hereditary material that they pass on in a stable manner. Producing and characterizing such GM mouse lines involves tremendous efforts. The number of GM mouse lines is increasing rapidly. Several limitations have to be considered when working with these animals: small colonies, the continuing danger of loss, often limited breeding success, the need to keep those mutants in stock, difficult and costly import procedures, and also the major (scientific) value of those mutants that are often available only with restrictions. Cryopreservation of pre-implantation embryos or of spermatozoa is a common approach to keep those mutant mouse lines available at any time, while dramatically reducing the need of living animals. Both strategies for cryopreserving embryos or spermatozoa have advantages and disadvantages, and the technique preferred is chosen case by case.

Cryopreservation of pre-implantation embryos is a very common method, consuming many embryo donors but leading to an easy and secure recovery. By contrast, mature spermatozoa for cryopreservation are available in most cases in unlimited numbers, and only a few donor animals are needed. However, following revitalization an *in vitro* fertilization (IVF) step is required, a complex approach not available for all genetic backgrounds. In this case, the animals obtained following revitalization are genetically dependent on the thawed spermatozoa and on the oocytes subjected to IVF (i.e., the genotype of origin will be lost). The quality of cryopreserved spermatozoa must be assessed as well as the IVF capacity of each line.[1–5]

Therefore, the revitalization capacity of cryopreserved material must be assessed. Although it is known that storage in the liquid phase of liquid nitrogen (LN_2) reliably maintains the viability of cryopreserved samples, storing conditions and duration of storage may affect the revitalization capacity.

To identify parameters affecting this capacity of the cryopreserved samples, for example, mouse embryos, additional assessment steps are necessary. For this assessment, the recovery-success rate of cryopreserved pre-implantation must be evaluated. Furthermore, embryos generated by IVF

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might be less viable than embryos received from donor females; this also needs to be investigated.

Another factor to be considered is the shipment of cryopreserved samples. The interchange of mutant mouse lines between different facilities is very common and reasonable, since the double generation and double characterization of a mutant line can be avoided. However, shipment of samples combined with handling may compromise their quality.

As published elsewhere, the genotype of the cryopreserved specimen is also preserved.[1,2,4] However, the genotype of the specimen remains to be assessed. For practical reasons, female wild type (WT) embryo donors are often bred with hemi- or heterozygous GM males. According to Mendelian laws, the outcome of that breeding should be 50% hemi- or heterozygous mutated embryos. However, a reduced viability due to the mutation and laboratory errors or mistakes might lead to a reduced outcome of the GM pups following cryopreservation. In addition, when applying IVF techniques using hemi- or heterozygous mutated males, a high frequency of mutant IVF related pups is not guaranteed. Subsequently, when using revitalized embryos it is mandatory to subject pups to an analysis of the genotype when assessing embryo transfers.[1,4]

Rodent facilities especially need to be protected against infections, so they are housed frequently in specified pathogen free (SPF) facilities. To keep the hygienic status at a high level, the importation of animals is strictly regulated; often only pups rederived (by embryo-transfer) are allowed to enter the target facility. In general, rodents and their embryos, germplasm, and ES-cells might be contaminated by microorganisms since these microorganisms can be reduced but not completely eliminated by extensive washing steps.[7,8] However, some viruses maintain the capacity to pass IVF and embryo-transfer procedures,[9–14] which is not the case for bacterial infectants.[15] Since frozen samples are frequently used for rederivation, care should be taken to prevent cross-contamination. Subsequently, investigations for possible contamination of the samples, the materials used, and the storage facilities are mandatory.

Here we describe our experience on the physical, genetic, and microbiological stability of the samples stored in repositories of GM mouse lines during the past 15 years.

Materials and Methods

Animal experimentation

All mice used in this study were housed in the animal facility of the German Cancer Research Center (DKFZ), Heidelberg, Germany. GM mouse lines originally received from different sources were bred and expanded *in-house,* whereas WT mice with corresponding genetic backgrounds (BDF, C3H, C57BL/6, CBA, DBA/2, FVB/N, NMRI) were received from Charles River (Sulzfeld, Germany). Individually ventilated caging systems (IVC) and barrier facilities (with open caging systems) were used as described in detail previously.[6] The age of the male mice used ranged between 3 and 9 months. Males were housed singly, and females were kept in groups of five. The health of the animals was monitored according to the Federation of European Laboratory Animal Science Associations (FELASA) recommendations.[16] Animal experimentation was performed according to the German Animal Welfare Act. All animal experiments were approved by the Animal Welfare Department of the Competent Authority (Regierungspräsidium Karlsruhe, Germany) and conducted under the surveillance of the intramural Animal Welfare Committee of DKFZ.

Criteria to select viable embryos

Embryos were selected microscopically (magnification 40X) as intact under the following conditions: round form, normal (not smaller or larger) size, normal cytoplasmic granulation, intact zona pellucida, the appropriate number of cells, not in another embryonic stage, not shrunk, and—so far as it can be detected microscopically—not dead.[3,4]

Cryopreservation of embryos

Cryopreservation of embryos was performed as described originally by Leibo,[2] with modifications described by Wayss et al.[3] and Schwab and Schenkel.[4] In brief: Morphologically intact embryos were transferred into ''freezing medium'' (1.5 M glycerol [Sigma-Aldrich, Taufkirchen, Germany] in M2 [Sigma-Aldrich], 1960–1980 mOsm). Following equilibration (10 min, on ice), the embryos were washed in ''freezing medium'' and intact embryos were selected for cryopreservation under the criteria mentioned above. Approximately 20 embryos were then loaded into a ''French straw'' (IMV Technologies, L'Aigle, France). A straw was filled with ''thawing medium'' (0.94 M sucrose [Sigma-Aldrich] in M2, 1700–1720 mOsm) over a length of 5 cm (ca. 100 μL) and separated by air with ''freezing medium'' for 1 cm (approx. 20 μL). Approximately 20 embryos were transferred in a very small volume into the ''freezing medium'' section using a glass capillary. Separated by air, one or two additional drops (approx. 10 μL each) of ''thawing medium'' were aspirated and both ends of the straw were sealed with an impulse tong sealer (Polystar 110 GE/150 D; Rische & Herfurth, Hamburg, Germany). Following 10 min equilibration on ice (0°C), the sample was placed into a control rate freezer at 0°C for 10 min and was afterwards frozen to −6°C with a cooling rate of 1 K/min. During a 10 min equilibration break, the liquid in the straw was seeded using liquid nitrogen (LN_2) cooled metal forceps. With a cooling rate of 0.4 K/min the embryos were frozen to −32°C, equilibrated for 10 min, and afterwards plunged directly into the liquid phase of LN_2.

Revitalization of embryos

Straws were transferred directly from LN_2 to room temperature according to Leibo.[2] To avoid an osmotic shock and to remove the glycerol from the embryos, both media within the straw were mixed by pushing into a Petri dish (tissue culture quality) immediately after thawing. Following repeated washing in M2, the revitalized embryos were selected by their morphology and were ready for further use.[3,4]

For imported embryos from other sources, the revitalization process was performed according to the sender's guidelines.

Cryopreservation of spermatozoa

Spermatozoa were cryopreserved according to the method of Ostermeier et al.[17] In brief, 3- to 9-month- old males were sacrificed. Spermatozoa were then collected from the epididymides and vasa deferentia. Spermatozoa were allowed to disperse from the tissue for 10 min at 37°C in cryoprotective media (CPM), 18% (w/v) raffinose (Sigma-Aldrich), 3% (w/v) skim milk (BD Diagnostics, Heidelberg, Germany), and

477 μM monothioglycerol (Sigma-Aldrich) in distilled water, and were loaded into 0.30 mL French straws (IMV Technologies). The straws were sealed with an impulse tong sealer, placed onto a polystyrene raft floating in LN_2 for at least 10 min, and then stored in LN_2. All GM donor males were subjected to re-genotyping.

In vitro *fertilization*

IVF was performed as described previously by Ostermeier et al.[17] and Diercks et al.[5] Donor females (3–4 weeks-old; 5 donors/IVF) with a genetic background according to the individual sperm donor were superovulated, as described elsewhere. These animals were sacrificed 12–14 h after the administration of human chorionic gonadotropin (Sigma-Aldrich) and the oocytes were removed from the swollen ampullae. Human tubal fluid (HTF) was used as IVF culture medium (Millipore MR-070-D; EMD Millipore). The medium drop was overlaid with mineral oil (Sigma- Aldrich) as described.[1,4] The IVF dishes (BD Diagnostics) contained one 500 μL HTF drop. Sperm samples were thawed at 37°C (in a water bath) for about 10 min. The spermatozoa in CPM were pushed out of the straw into the HTF drop and were incubated for 1 h at 37°C. Co-incubation of oocytes and spermatozoa in HTF lasted 5–6 h. The resulting zygotes were washed and incubated overnight in a 200 μL drop of potassium simplex optimization medium (KSOM; Millipore MR-020P-5F; EMD Millipore, Schwalbach, Germany). Next, the proportion of two-cell embryos was calculated. These embryos were subsequently ready for further use.

Embryo transfer

Revitalized pre-implantation embryos were transferred into the oviduct of day 0.5 or day 2.5 vaginal plug positive (VP^+) - pseudo pregnant foster mothers by standard procedures.[1] A successfully completed revitalization was acknowledged when living animals were received. The morphological criteria described above were sufficient to decide whether the revitalization was successful.[3,4]

ES cells

Large-scale production of C57BL/6 ES cell clones was performed as described by Skarnes et al.[18] The construction of modular gene-targeting vectors was performed in high-throughput using BAC recombineering in 96-well format. For targeting in C57BL/6N ES cells,[19] indexed C57BL/6J BAC libraries were used.[20] Electroporation of the targeting vectors in C57BL/6N ES cells was performed in 5 x 5 multi-well cuvettes. Drug-resistant colonies were picked and expanded to two DNA copies for genotyping and four copies for long-term storage in vapor phase liquid nitrogen. Correctly targeted clones were determined by long-range PCR (LR-PCR) over the 5' and 3' homology arm of the targeting cassette. For the production of mutant mouse lines, ES cells were expanded from frozen stocks. ES cell DNA was used to perform quality controls such as the verification of the LR-PCR results and TaqMan® (Life Sciences, Thermo Fisher Scientific, Schwerte, Germany) copy number assays for key elements of the targeting cassette. Quality control positive clones were again expanded, single cells were produced, and microinjection into BALB/c mouse blastocysts was performed. Furthermore, ES cell clones of unknown origin were made available by several research groups in Heidelberg and Basel.

Genotyping

Biopsies of the animals were taken (e.g., the tip of the tail), DNA was extracted, and the genotype determined using standard procedures such as Southern blots or PCR. The genotyping was performed by the group responsible for the individual mouse line.[1]

Storage of samples

Cryopreserved samples were stored in the liquid phase of LN_2. Storage of the straws was performed according to Schwab and Schenkel.[4] The following permanent freezers were used: Thermo Electron Cryo 200 (Thermo Fisher Scientific) and Messer Chronos Biosafe (Cryotherm, Kirchen/Sieg, Germany). The permanent freezers located in Heidelberg were cleaned before use: all inside surfaces were completely sprinkled with Biocidal ZF™ (WAK-ZF-1 WAK-Chemie, Steinbach, Germany) and incubated with a closed lid over night at room temperature. Afterwards, this step was repeated with an open lid. Finally, all surfaces were rinsed with isopropanol, and after complete drying the freezer was ready for further use.

Microbiological investigations

For virological testing, DNA was extracted from ES-cells, spermatozoa, and embryos with the QIAsymphony SP instrument using the DSP virus/pathogen mini kit and the complex 400 protocol (Qiagen, Hilden, Germany). RNA was extracted with the MagNA Pure 96 System (Roche, Penzberg, Germany). Multiplex PCR for DNA viruses was performed as described in detail by Höfler et al.[21] and accordingly also for RNA viruses. Tests were performed to detect Mycoplasma spp. and the following murine viruses: M*urine adenovirus 1 and 2 (MAdV-1 and 2), Ectromelia virus (ECTV), Murine pneumotropic virus (MPtV, K-virus), Murine polyomavirus (MPyV), Parvoviruses (NS1), Minute virus of mice (MVM), Mouse parvovirus (MPV), Lactate dehydrogenase elevating virus (LDV), Lymphocytic choriomeningitis virus (LCMV), Mammalian orthoreovirus 3 (Reo 3), Murine hepatitis virus (MHV), Murine norovirus (MNV), Murine pneumonia virus (PVM), Murine Rotavirus A (EDIM), Sendai virus (SeV), Theiler's murine encephalomyelitis virus (TMEV), Encephalomyocarditis virus (EMCV), Hantaan Virus (HTNV), Puumala virus (PUV)*, and *Seoul virus (SEUV)*.

Samples for bacterial testing were taken from the lid (middle and rim of the Styrofoam isolation; hinged and fully removable lids) and the seam of the permanent freezer, summarized as ''top,'' or the physical bottom of permanent LN_2-freezers or the goblets, summarized as ''bottom,'' and from dry-shippers under sterile conditions by using disposable uterine sterile culture swabs for mares (#290955 Equivet Kruuse, Langeskov, Denmark). In the presence of LN_2 sampling was possible with frozen swabs as well. Swabs were streaked on blood agar, MacConkey agar, and Kimmig agar, and subsequently forwarded under sterile conditions into a tube with liquid culture medium (Standard I broth, Merck, Darmstadt, Germany). Bacterial cultures were incubated for at least 5 days at 30°C.

Goblets were removed from the permanent freezer and stored in a fume hood with negative air-pressure until the

LN_2 was evaporated. The remaining aqueous liquid was forwarded to bacterial culture.

The survival time of bacteria was tested by soaking a filter paper disc with 25 μL of an overnight culture and after drying putting it into LN_2 for 2 days. Very commonly found gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*Citrobacter freundii*) were selected as representatives. Survival of bacteria was tested by incubating the filter disc in liquid culture medium and streaking on solid media after incubation at 37°C for 24 h.

Statistics

A possible significance between two groups of data was calculated by a Student-Newman-Keuls Test; $p < 0.05$ is significant, $p < 0.001$ is highly significant.

Results

In order to understand the possible risks of handling or storing cryopreserved samples, this study analyzed the revitalization capacity, in part after long-term storage, the genotypes of mutant animals, and the microbiological status of the materials used.

Revitalization rate

10,942 pre-implantation embryos recovered by flushing oviducts (8-cell stage) were cryopreserved, stored *in-house* in 551 straws, and revitalized as described above. When investigating the morphology of these embryos, 10,264 or 93.82% of all embryos were shown to be viable. If embryos, received from IVF, overnight culture, and subsequent cryopreservation in the two-cell stage, were subjected to the recovery process, out of 421 cryopreserved embryos (stored in 27 straws) 349 or 82.93% of all embryos were viable. In the case of 765 imported pre-implantation embryos (two- or eight-cell stages, stored in 25 tubes of different origin) 564 were revitalized (i.e., 73.73% of all embryos seemed to be viable) (Fig. 1). The recovery rate of the embryos stored *in house* was significantly higher than the recovery rate of IVF related embryos ($p = 0.001$) or imported embryos ($p = 0.03$).

Storage period

To study the effects of different durations of storage, 3848 cryopreserved pre-implantation embryos were revitalized. They had been stored in 178 straws in the liquid phase of LN_2 for a period between 0 and 158 months. Then we looked at the percentage of viable embryos in relation to the storage period. However, there was no significant effect of the storage period detectable (Fig. 2).

Genotypes of revitalized samples

When cryopreserving mutants, it is mandatory to verify the genotype of the frozen samples. The cryopreservation of embryos produced by hemi- or heterozygous mutated GM males mated to WT females is very common. According to Mendelian laws, in this case 50% mutants are expected. However, when breeding homozygous mutated GM males with homozygous mutated GM females of the same line, all embryos produced maintain the mutation on both alleles. To assess the genotypes of randomly selected cryopreserved specimens, revitalized embryos were subjected to embryo-transfer, and the pups received were subjected to genotyping (Fig. 3). Out of 508 pups (one half are expected to carry the mutation), 209 or 41.14% were mutant animals. The origins of 220 pups were from transgene overexpressing lines, 13 from spontaneous mutants, and 275 from homologous recombinants. In the case

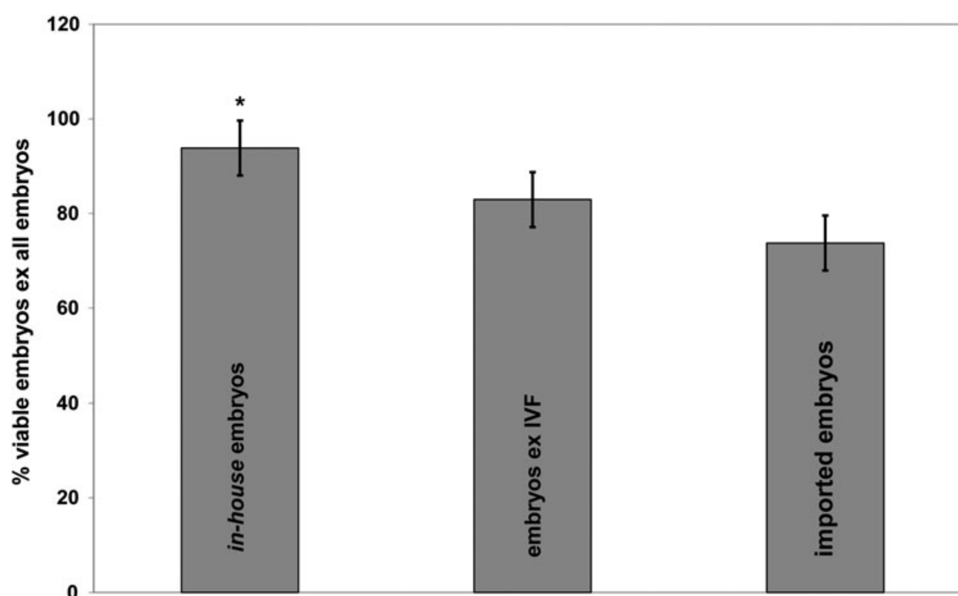

FIG. 1. Revitalization capacity: From 10,942 pre-implantation embryos cryopreserved and stored *in house* in 551 straws, 10,264 or 93.82% showed an intact morphology following revitalization. From 421 embryos (stored in 27 straws), received from IVF and subsequent cryopreservation, 349 or 82.93% showed an intact morphology following revitalization. From 765 imported pre-implantation embryos (stored in 25 tubes of different sources), 564 or 73.73% showed an intact morphology following revitalization. The recovery rate of the embryos stored in house was significantly higher (asterisk) than the recovery rate of IVF related embryos ($p = 0.001$) or imported embryos ($p = 0.03$). For further details see main text. (% viable embryos ex all embryos = % (portion) of viable embryos out of all embryos found after thawing).

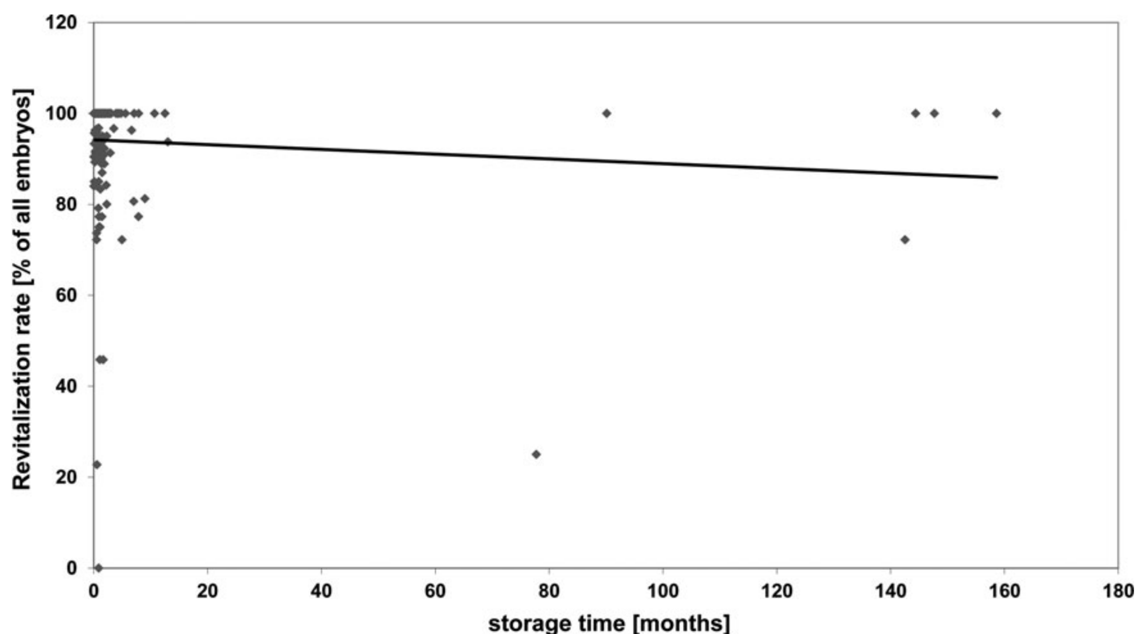

FIG. 2. Storage period: 3848 cryopreserved pre-implantation embryos were stored between 0 and 158 months and revitalized. Each *dot* shows the percentage of morphologic intact embryos out of all embryos of the corresponding sample.

of 317 pups expected to be all mutated (homozygous mutated GM males used for breeding in embryo production), only 303 or 95.58% were genotyped as mutant animals. The origins of 210 pups were homologous recombined lines, 16 spontaneous mutants, and 91 transgene overexpressing lines. Here, in the case of seven transfers of embryos from homologous recombinants, the number of GM offspring was not as expected. However, out of 27 pups received from embryos generated by IVF without exact forecast of a percentage of mutant offspring, 16 pups or 59.25% were transgenic. This leads to the conclusion that not all males used for embryo production were correctly genotyped.

Hygienic quality

Infectious agents might be co-preserved within the cryopreservation process or the infectious agents enter the permanent freezers by the transport or storage of contaminated

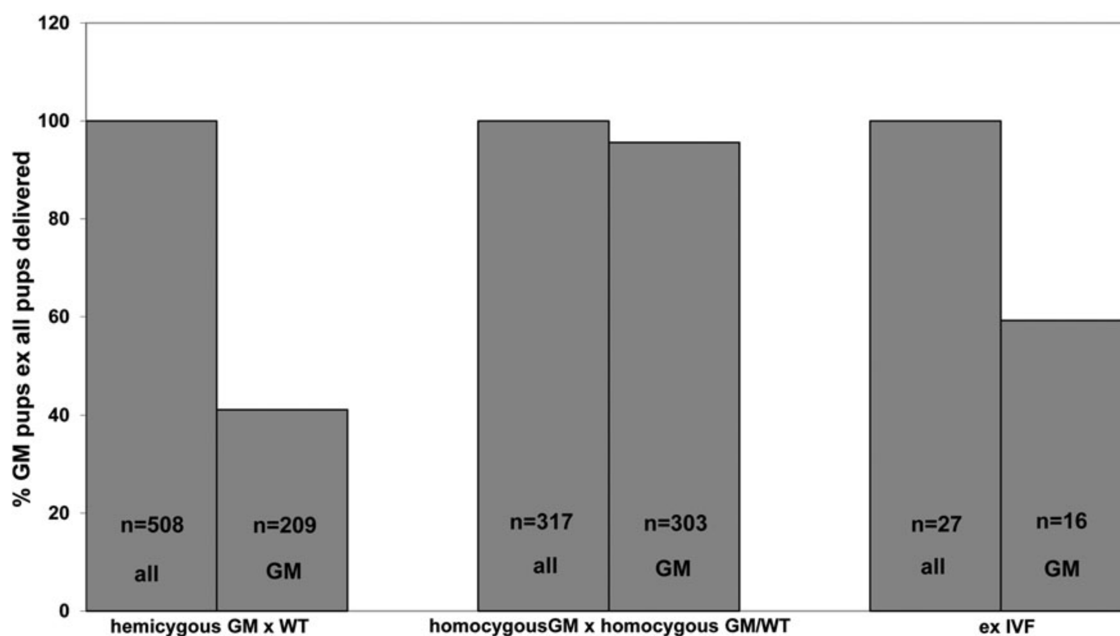

FIG. 3. Genotypes of off-springs from revitalized samples: From 508 pups (from mating WT x hemi-/ heterocygously mutated GM animals) 209 or 41.14% were GM animals. From 317 pups expected to be all mutated (embryos from WT x homocygously mutated GM animals) 303 or 95.58% were GM animals. From 27 pups received from embryos generated by IVF 16 pups or 59.25% were GM animals. For further details see main text. (% GM pups ex all pups delivered = % (portion) of GM pups out of all pups delivered following embryo-transfer as tested by PCR or Southern blotting.)

Table 1. Hygienic Stability

	Environmental microorganisms	*Mouse pathogens*
Media used	–	–
LN_2 freezer, top $n=20$	+	nd
LN_2 freezer, bottom $n=20$	+	nd
Dry shipper $n=15$	+	nd
Gas phase permanent freezer	nd	nd
Room air $n=4$	+	nd
Spermatozoa $n=80$	–	–
Embryos $n=28$	–	–
ES-cells $n=106$	–	–
Off-springs after embryo-transfer	–	+

Qualitative determination of infections; "+" = presence of microorganisms or mouse pathogens proven; "–" = not detected; "nd" = not done. "Top" includes all parts of the lid and the seam of the freezer; "bottom" the physical bottom and the goblets. The numbers mentioned for embryos, spermatozoa, and ES-cells indicate the number of samples investigated. For details see main text.

material. To verify a possible capacity to enter the LN_2, *Citrobacter freundii* and *Staphylococcus aureus* samples were subjected on filter papers into LN_2. Afterwards, sterile filters were transferred into the same container. Following evaporation of the major LN_2-fraction, the originally sterile filters were forwarded to a liquid culture medium and incubated overnight. Both bacterial species survived in LN_2 without cryoprotectant and grew in the medium (data not shown in detail).

To get an overview of possible risks and pathways of infectious microorganisms, possible contamination steps during the whole cryopreservation, storage, and recovery process were investigated. 28 straws containing 480 pre-implantation embryos of ten GM mouse lines (stored in LN_2 8 to 17 years), 80 straws containing spermatozoa of eight GM mouse lines (stored in LN_2 approx. 3 years), and 106 mouse ES-cell samples of different origin (stored in LN_2 up to 15 years) were assessed.

As shown in Table 1, microorganisms or mouse pathogens were never found in closed vials with samples of embryos, spermatozoa, or ES cells, nor in cryo-tubes, culture-media, or buffers used. By contrast, environmental organisms were often detected in all parts of the freezers, even if these parts were covered with LN_2, or in dry shippers of different sources. Samples taken from permanent freezers and from surfaces of dry shippers were regularly contaminated with molds, yeasts, aerobic spore forming bacteria, gram-positive cocci, and occasionally also with *Enterobacteriaceae* and other gram-negative bacteria (not further characterized). Similar microorganisms were detected in the room air as well. Due to technical problems, the vapor phase of LN_2 could not be investigated. However, during the further experimental steps to recover a line, dangerous mouse pathogens were detected in seven of 554 litters following embryo-transfer. Details and reasons for this finding remain to be investigated.

Discussion

Cryopreservation is a very valuable tool to save mutant mouse lines, to protect them against loss, and to reduce the number of laboratory animals used to keep a line available. If a sufficient number of embryos or of spermatozoa is cryopreserved, a line must not be kept by continuous breeding. However, the interchange of mutant mouse lines between different laboratories is very common and makes sense, double-generations or -characterizations can be avoided when using an established line. Subsequently, shipments are done frequently. Shipments of cryopreserved samples are much easier than shipments of living animals. Due to the importance of cryopreserved samples, a thorough assessment of the quality and a safe long-term storage strategy are mandatory. Possible factors negatively influencing this stability are to be elucidated and subsequently eliminated or at least considered.

Recovery of mouse lines

Stable storage is a prerequisite to recover a line. One has to take into consideration that cryopreserved samples have a volume of a few microliters, thawing within a few seconds at room temperature. The physicochemical stability of water molecules, required for storage stability, is only given at a temperature of less than $-130°C$. Subsequently, all handling steps might represent a possible danger for the stability of those samples.

Revitalization of randomly selected cryopreserved samples is a common approach to assess these. As published earlier,[3–5] the proper morphology is an important parameter to determine the revitalization rate.

As shown in Figure 1, eight-cell embryos cryopreserved and stored *in-house* exhibit a high recovery success rate. Out of more than 10,000 embryos, nearly 94% maintained the capacity to recover proper morphology. Even a storage period of up to 15 years did not influence the revitalization rate (Fig. 2).

Additional assessment steps are required to acquire complete information (e.g., the recovery-success rate of embryos cryopreserved in different pre-implantation stages, or the revitalization rate of imported embryos). However, all these additional assessment steps are failure-prone and might be therefore excluded in favor of a storage assessment.

Our and other's data show that the cryopreservation of higher embryonic pre-implantation stages results in a higher revitalization rate (i.e., two-cell stages versus eight-cell stages). If two-cell embryos generated by IVF and cryopreserved following overnight culture were investigated, the revitalization rate was only about 83%.[1–4] This might be a result of the lower developmental stage used for cryopreservation, or a possible two-cell-block due to IVF and *in vitro* culture. Hence, this reduced developmental capacity is covered by high yields of IVF-related embryos.

However, much more impressive is the significantly lower developmental capacity of imported embryos (i.e., embryos exposed to repeated handling steps and shipment). Since most facilities cryopreserving mutant mouse lines are experienced and have a very high quality standard, we think that the reduced developmental capacity results from shipment and handling, out of the control of the cryopreserving facility. An undiscovered thawing and subsequent uncontrolled re-freezing of a sample would result in the complete loss of the capacity to revitalize. Therefore, the procedures of carriers, handling by authorities, speed of transport, etc. should be assessed and improved whenever possible. In our hands, an extensive preparation of a shipment including all administrative steps can reduce the travel time significantly.

Genotypes

The aim of cryopreservation is not only to retrieve a mouse. There is also the need to recover the mutation reliably. In many cases the most productive approach is to produce embryos by mating GM males (frequently hemi- or heterozygous mutated) with WT female embryo donors. This maintains the advantage to have a sufficient number of females available qualifying for optimum superovulation and high embryo rates. On the other hand, due to Mendelian laws, this strategy theoretically results in 50% GM and 50% WT embryos and subsequent progeny. If the mutation leads to a reduced viability, the portion of GM progeny might be reduced. Errors in genotyping (particularly the donor males) or mistakes in the facility will also reduce this portion. When analyzing the outcome of genotyping the offspring of embryo-transferred foster mothers, a 42% frequency of GM offspring was discovered (Fig. 3). This might be sometimes also due to odd sizes of the offspring and a very few cases of the influence of the mutation. The major danger might be the use of false-genotyped or confounded animals. This suspicion was substantiated when using males genotyped as homozygous mutated. Here, mating with any female must result in 100% GM progeny. We discovered only 95.58%, indicating an error. To avoid similar mistakes, all males used for the production of embryos must be re-genotyped. A portion of more than 50% GM progeny following IVF shows that this approach might result in a sufficient hereditary efficiency. However, the spermatozoa donors must be subjected to re-genotyping, too, and the capacity to recover the mutation following IVF must be proven.

Hygienic quality

Colonies of laboratory rodents are highly prone to infections[7,9] due to frequent introduction of animals and biological materials of rodent origin, and frequent access of personnel. Once introduced, agents are usually difficult to eradicate by medication, and in most cases the unit must be closed and disinfected and the lines originally housed there must be rederived and re-established. This results in tremendous costs, delays, and consumption of animals. Powerful strategies are required to prevent contamination of a facility. Since most surveillance programs deal with sentinels, cryopreservation techniques are used to rederive infected mouse lines. Due to the fact that handling and storage of the samples are not under (fully) sterile conditions, possible pathways of contamination must be discovered.

Surprisingly, it was proven in a preliminary experiment that microorganisms such as *Citrobacter freundii* and *Staphylococcus aureus* have the capacity to survive in LN_2 without passing a correct cryopreservation process. This addresses the question of whether environmental organisms can contaminate stored samples and accumulate in permanent freezers. Morris[22] published data proving the accumulation of environmental organisms in permanent freezers, confirming our data. We think that, depending on the handling of the freezers, aspiration of room air during the opening of the permanent freezer will also aspirate microorganisms. They stick in most cases in the water crystals located at the top, the lid, and the seam of the permanent freezer. Due to the room humidity, these crystals grow and fall randomly to the bottom of the container and they potentially are present as agents maintaining their viability. Hence, microorganisms will accumulate in the liquid phase of LN_2 at the deepest part of the container; this was proven by our data, too, when investigating permanent freezers and dry shippers of different sources. It is rather implausible that the density of microorganisms in the liquid phase of LN_2 is high enough to contaminate improperly closed tubes. However, Clarke,[23] Bielanski and Vajta,[24] Bielanski et al.,[25] and Kuleshova and Shaw[26] describe the risk of contamination when using incorrectly sealed tubes. We have never observed this type of contamination, but we take care that all samples are properly closed (by using a sealer). Bielanski et al.[27] or Kyuwa et al.[28] did not detect any microorganism passing safely sealed tubes. The alternative storage in the gas phase of LN_2 might reduce the risk of a possible cross-contamination; however, storage in the gas phase might reduce the physical stability of the samples when using straws with 20 μL content. In addition, contradictory data are published about possible contamination of the gas phase of LN_2.[29,30] We also cross-checked all media used after the last washing step and never found any microorganisms. This finding agrees with the data published by Agca et al.[7] showing that intensive washing steps can reduce the content of microorganisms.

Long-term stored samples of ES-cells, pre-implantation embryos, and spermatozoa were investigated to prove or exclude mouse pathogens. However, there is no doubt that samples may be contaminated prior to freezing and may therefore maintain the contamination, also with unwanted microorganisms, during storage. As described by Nicklas and Weiss,[31] biological material maintains the potential to forward pathogenic material. As shown here, we were unable to detect mouse specific viruses in ES-cells. This agrees with the study of Nicklas and Weiss,[31] but disagrees with the data of Kyuwa.[8] Embryo-transfer is a proven technique to rederive embryos, but this technique might fail, especially in case of viral infections.[9,11–14] To improve the rederivation effect, some reports or handbooks recommend the treatment of embryos with proteases.[32,33]

Taken together, there are many possibilities to accumulate microorganisms in permanent freezers and dry shippers. The samples must be protected against these and details remain to be elucidated. We never detected mouse pathogens in our samples, but sometimes this occurred after embryo-transfer. We think that the contamination rate is very low, but high enough to represent a major danger for a target facility. Therefore it is important to reduce the contamination rate, especially of microorganisms, and to keep the transferred foster mothers in a special quarantine facility until the success of the rederivation is confirmed. Improved storage strategies might be developed. This approach is costly and time consuming, but much better than having to disinfect and to reestablish a mouse facility.

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Author Disclosure Statement

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